

## Glyceraldehyde-3-Phosphate Dehydrogenase of *Streptococcus pneumoniae* Is a Surface-Displayed Plasminogen-Binding Protein

Simone Bergmann,<sup>1</sup> Manfred Rohde,<sup>2</sup> and Sven Hammerschmidt<sup>1\*</sup>

Research Center for Infectious Diseases, University of Würzburg, Würzburg,<sup>1</sup> and GBF-German Research Centre for Biotechnology, Braunschweig,<sup>2</sup> Germany

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**The recruitment of plasminogen endows the bacterial cell surface of *Streptococcus pneumoniae* with proteolytic activity. In this study we demonstrate specific plasmin- and plasminogen-binding activity for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is located in the cytoplasm as well as on the surface of pneumococci. GAPDH exhibits a high affinity for plasmin and a significantly lower affinity for plasminogen.**

A prerequisite for the invasiveness of a pathogen is the pathogen's ability to breach epithelial as well as endothelial barriers in order to gain access to the submucosa and blood. A successful strategy used by pathogenic bacteria to degrade the extracellular matrix and to promote invasiveness is the recruitment of proteolytic activity to the bacterial cell surface (19, 21). *Streptococcus pneumoniae*, a common etiologic agent of respiratory tract diseases and life-threatening invasive diseases, is able to capture plasminogen on the bacterial cell surface. Subsequent activation by tissue-type and urokinase-type eukaryotic plasminogen activators allows the bacteria to acquire surface-associated proteolytic activity (10, 17). Plasminogen, a glycoprotein, is the zymogen of the serine protease plasmin, which is a key enzyme of the fibrinolytic pathway (7). The acquired plasmin activity promotes dissemination and transmigration of the pathogen through reconstituted basement membranes (6, 10, 21).

Analysis of plasminogen binding to viable pneumococci by the method described by Scatchard (25) indicated the presence of more than one plasminogen receptor on the pneumococcal cell surface. A blot overlay with soluble radioiodinated human plasminogen showed binding to eight pneumococcal components (2).  $\alpha$ -Enolase (Eno) was identified as the major plasminogen- and plasmin-binding protein of *S. pneumoniae* (2). A further peptide sequence, obtained by an N-terminal sequence analysis of excised proteins positive for plasminogen binding, showed 100% homology to the N-terminal sequence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GapA) in *S. pneumoniae* strains (<http://www.tigr.org/tigr-scripts/CMR2/CMRGenomes.spl>) (16, 26). These results identified the GAPDH of *S. pneumoniae*, which is an essential enzyme of the glycolytic pathway, as a putative plasminogen-binding protein. For expression cloning of *gapA*, the gene was amplified by PCR using the chromosomal DNA of *S. pneumoniae* serotype 2

(ATCC 11733) and oligonucleotide primers SB 13 (5'-GGAT CCTTGCTTGACACTTGTGAAATAC-3'), incorporating an in-frame BamHI restriction site at the 5' end, and SB 14 (5'-AAGCTTTTATTAGCAATTTTTCGAAGTATT-3'), incorporating an in-frame HindIII restriction site at the 3' end. The BamHI- and HindIII-digested PCR product was cloned into the similarly digested expression vector pQE30 (Qiagen). The purification of His-tagged GAPDH under native conditions was conducted according to a standard protocol (Qiagen). In order to identify the subcellular localization of GAPDH by immunoblot analysis, polyclonal antibodies against purified recombinant GAPDH were raised in rabbits by routine immunogenic procedures (Eurogentec). Results indicated the presence of GAPDH in both the cytoplasmic fraction and the cell surface protein fraction of *S. pneumoniae* (Fig. 1). A sequence comparison performed with the algorithm of Lipman and Pearson (18) revealed 89.0% identity to the group A streptococcal GAPDH Plr/streptococcal surface dehydrogenase and 85.2% identity to GapC in *Streptococcus equisimilis* (14, 20, 24). Plasminogen- and plasmin-binding activity, which is most likely mediated via C-terminal lysyl residues (28), to both streptococcal proteins has been reported. The pneumococcal GAPDH contains two C-terminal lysine amino acids which are separated by isoleucine and alanine. The C-terminal sequences (>40 amino acids) of the above-mentioned GAPDH proteins are 100% identical. Moreover, sequence comparison revealed identical C termini in the GAPDHs of *Streptococcus agalactiae*,



FIG. 1. Identification of the cellular distribution of the pneumococcal GAPDH. Immunoblot analysis, using anti-GAPDH antibodies, of the cell wall fraction (lane 1) and soluble fraction (lane 2) of *S. pneumoniae* R6x and of the soluble fraction of an *S. pneumoniae* *eno* internal deletion mutant expressing  $\alpha$ -enolase with mutated plasminogen binding sites (lane 3).

\* Corresponding author. Mailing address: Research Center for Infectious Diseases, University of Würzburg, Röntgenring 11, 97070 Würzburg, Germany. Phone: 0049-931-312153. Fax: 0049-931-312578 E-mail: s.hammerschmidt@mail.uni-wuerzburg.de.

TABLE 1. Comparison of GAPDH sequences and C-terminus identities among different streptococcal species

Species	Protein	% Identity	C-terminal sequence	Accession no.	Reference(s)
<i>S. pneumoniae</i>	GapA	100	VRTLEYFAKIAK	AJ505822	This paper
<i>S. pyogenes</i>	Plr/SDH	89.0	VRTLEYFAKIAK	M95569	20, 24
<i>S. agalactiae</i>	GapC	86.4	VRTLEYFAKIAK	AF421899	13
<i>S. dysgalactiae</i>	GapC	89.6	VRTLEYFAKIAK	AF375662	EMBL
<i>S. equisimilis</i>	GapC	85.2	VRTLEYFAKIAK	X97788	14
<i>S. mutans</i>	GapC	88.7	VRTLEYFAKIAK	AE014883	1

*Streptococcus dysgalactiae*, and *Streptococcus mutans* (Table 1). Interestingly, the GAPDHs of streptococci of groups A, C, and G were recently identified as extracellular targets for a broad spectrum of extracellular matrix proteins and especially for plasmin and plasminogen (20, 22).

In order to demonstrate the plasminogen-binding activity of the pneumococcal GAPDH, total proteins of the serotype 2 strain and the serotype 2 *eno* internal deletion (*eno*<sup>int/del</sup>) mutant were incubated with immobilized plasminogen. The mutant expresses an  $\alpha$ -enolase with a deletion of the C-terminal lysyl residues and amino acid substitutions in the internal plasminogen-binding motif of Eno. The *Eno*<sup>int/del</sup> mutant exhibited substantially reduced plasminogen-binding activity (3) and was used in order to avoid binding of the  $\alpha$ -enolase to plasminogen. Plasminogen (obtained from Sigma or provided by K. Preissner, Giessen, Germany) was immobilized on a polyvinylidene fluoride membrane, and GAPDH binding was detected with polyclonal anti-GAPDH antiserum followed by horseradish peroxidase-conjugated anti-rabbit antibody. Results demonstrated a concentration-dependent GAPDH-plasminogen interaction, in which the protein-protein interaction depends on both the amount of immobilized plasminogen and the amount of soluble GAPDH (Fig. 2).

In an attempt to visualize the subcellular localization of GAPDH and the binding of plasminogen to GAPDH protein, preembedding, labeling studies were carried out as recently demonstrated for Eno (2). The unencapsulated pneumococcal strain R6x and the encapsulated pneumococcal strains of serotypes 2 (ATCC 11733) and 35A (NCTC 10319) were pre-labeled with polyclonal protein A-purified anti-GAPDH immunoglobulin G and 15-nm-diameter gold particles coupled to protein A before embedding. Ultrathin sections revealed that the GAPDH of *S. pneumoniae* resembles the  $\alpha$ -enolase located on the bacterial cell surface of unencapsulated (Fig. 3B) and encapsulated (Fig. 3A and C) strains. These results indicate that the common surface disposition of GAPDH is indepen-

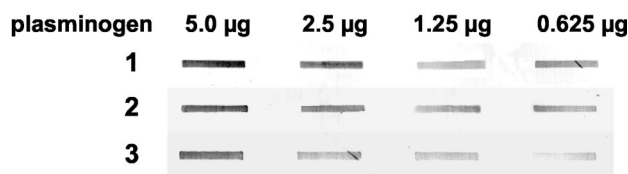


FIG. 2. Binding of pneumococcal GAPDH to plasminogen. Human plasminogen was immobilized in the different amounts indicated. GAPDH was purified under native conditions from the cytosolic compartment of the serotype 2 *eno*<sup>int/del</sup> mutant pneumococci (ATCC 11733). Rows 1 to 3, binding of GAPDH applied in serial dilutions (1:2).

dent of the state of capsular polysaccharide expression. The surface-located glycolytic enzymes of the Embden-Meyerhof-Parnas pathway and other surface-displayed proteins like PavA of *S. pneumoniae* (15) and FBP54 of *Streptococcus pyogenes* (8, 9) lack the classical secretion and anchoring mechanisms (11, 12, 27, 29) and thus constitute a novel class of exported proteins of gram-positive bacteria (5). The conditions and factors required for protein secretion, as well as the mechanism of anchoring these proteins, are not known. How plasminogen is bound to GAPDH or Eno through the capsule also remains unknown.

The dynamics of GAPDH-plasminogen and GAPDH-plasmin complex formation and their dissociation were analyzed by surface plasmon resonance (SPR) technique. GAPDH was covalently immobilized on a BIAcore CM5 sensor chip as previously described (3, 23). The association and dissociation kinetics of Glu-plasminogen and plasmin (Sigma) to GAPDH were evaluated according to the heterogeneous ligand model ( $A + B1 \leftrightarrow AB1$ ;  $A + B2 \leftrightarrow AB2$ ). This revealed two equilibrium constants; for plasminogen,  $K_{D1}$  was equal to  $4.3 \times 10^{-7}$  M and  $K_{D2}$  was equal to  $1.6 \times 10^{-10}$  M, and for plasmin,  $K_{D1}$  was equal to  $2.8 \times 10^{-8}$  M and  $K_{D2}$  was equal to  $5.2 \times 10^{-8}$  M. Studies using SPR showed that the pneumococcal GAPDH had a higher affinity for the serine protease plasmin than for its zymogen plasminogen (Fig. 4), as has also been shown previously for the Plr/streptococcal surface dehydrogenase of group A streptococci (20). The differences between

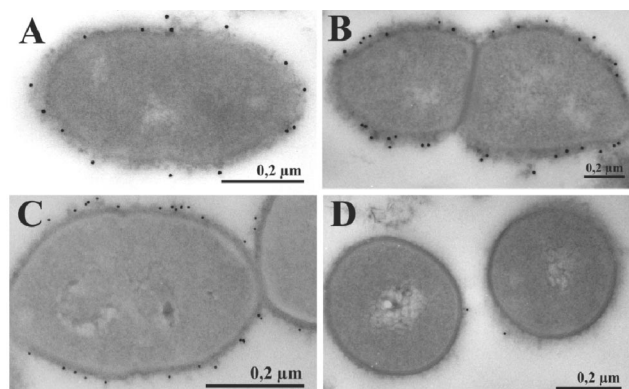


FIG. 3. Immunoelectron microscopic visualization of GAPDH on the surface of *S. pneumoniae* ATCC 11733 (type 2) (A), unencapsulated R6x (B), and *S. pneumoniae* NCTC 10319 (type 35A) (C). (A to C) GAPDH was visualized on the bacterial cell surfaces of ultrathin sections of preembedding labeled samples by using anti-GAPDH antibodies and protein A-15-nm-diameter gold particle studies. (D) Visualization of unspecific binding of protein A-15-nm-diameter gold particles to type 2 pneumococci.

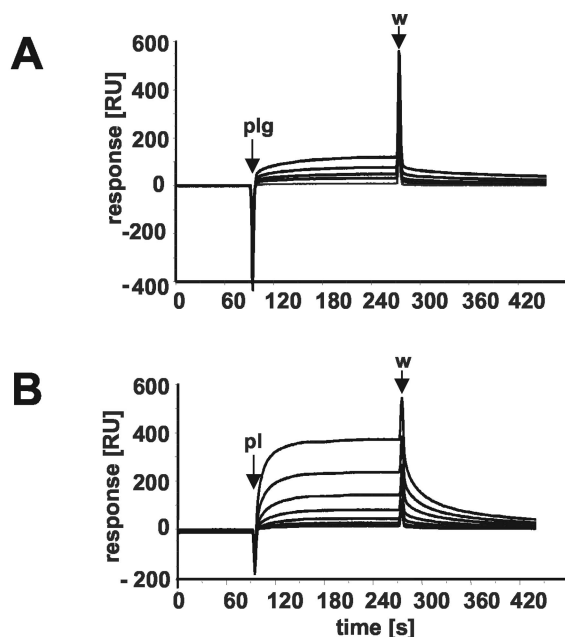


FIG. 4. SPR measurements of GAPDH-plasminogen (A) and GAPDH-plasmin (B) interactions. Purified GAPDH protein was immobilized on a BIAcore CM5 sensor chip, and plasmin and plasminogen were used as the analyte. Binding kinetics and concentration dependence of binding of plasminogen and plasmin to GAPDH are shown as changes in plasmin resonance. Plasmin and plasminogen were used in concentrations of 500 (for plasminogen only), 250, 125, 62.5, 31.25, 15.625, 7.8, and 3.9 nM. The blank run was subtracted from the sensorgram. Pl and Plg, starts of injection; w, stop of injection; RU, relative units.

equilibrium constants are probably the result of conformationally dependent structure recognition by Plr of the plasmin or plasminogen molecule (4). The interaction of GAPDH and plasminogen produced lower equilibrium constants than the Eno-plasminogen dissociation and complex formation did ( $K_{D1}$ ,  $8.6 \times 10^{-8}$  M;  $K_{D2}$ ,  $5.5 \times 10^{-10}$  M) in *S. pneumoniae* (3), indicating a lower affinity of GAPDH for plasminogen. Pathogenic bacteria acquire proteolytic activity by the recruitment and subsequent activation of plasminogen. A recent study indicated that the nonglycolytic property of  $\alpha$ -enolase contributes to the pathogenesis of pneumococci (3). The concerted action of both  $\alpha$ -enolase and GAPDH on the bacterial cell surface might, therefore, result in an enhancement of the pathogen's ability to degrade the extracellular matrix and to invade host tissues.

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